Oxysterols Induce Differentiation in Human Keratinocytes and Increase Ap-1-Dependent Involucrin Transcription

Karen Hanley,* Dean C. Ng,*† Shan Shan He,* Peggy Lau,* Katherine Min,* Peter M. Elias,* Daniel D. Bikle,*† David J. Mangelsdorf,‡ Mary L. Williams,§ and Kenneth R. Feingold*†

Departments of *Dermatology, †Medicine, §Pediatrics, University of California, San Francisco, California, Howard Hughes Medical Institute, and Department of Pharmacology, ‡University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, and the Dermatology and Medical Services Department of Veterans Affairs Medical Center, San Francisco, California, U.S.A.

Ligands and activators of the nuclear hormone receptor superfamily are important in the regulation of epidermal development and differentiation. Previously, we showed that naturally occurring fatty acids, as well as synthetic ligands for the peroxisome proliferator-activated receptor, induce keratinocyte differentiation *in vitro*. Here we asked whether oxysterols, another class of lipids formed *de novo* in the epidermis and that activate liver X-activated receptor, regulate keratinocyte differentiation. mRNA and protein levels of involucrin and transglutaminase 1, markers of differentiation, increased 2- to 3-fold in normal human keratinocytes incubated in the presence of 25- or 22R-hydroxycholesterol in low calcium. In high calcium, which alone induces differentiation, mRNA levels were further increased by oxysterols. Rates of cornified envelope formation, an indicator of terminal differentiation, also increased 2-fold with oxysterol treatment. In contrast, the rate of DNA synthesis was inhibited approximately 50% by oxysterols. Transcriptional regulation was assessed in keratinocytes transfected with either transglutaminase 1 or involucrin promoter-luciferase constructs. 22R-hydroxycholesterol increased transglutaminase 1 and involucrin promoter activity 2- to 3-fold. Either deletion of the –2452 bp to –1880 bp region of the involucrin promoter, or mutation of the AP-1 site within this region, abolished oxysterol responsiveness. Moreover, increased AP-1 DNA binding was observed in oxysterol-treated keratinocytes by gel shift analyses. Finally, we demonstrated the presence of liver X-activated receptor α and β mRNAs, and showed that oxysterols stimulate a liver X-activated receptor response element transfected into keratinocytes. These data suggest that oxysterols induce keratinocyte differentiation, in part through increased AP-1-dependent transcription of the involucrin gene, an effect that may be mediated by liver X-activated receptor. ***Key words: cornified envelope/LXR/nuclear receptor. J Invest Dermatol 114:545–553, 2000***

The epidermis is a stratifying keratinizing epithelium, with its uppermost layer, the stratum corneum, providing the skin with structural integrity and a barrier to excessive water loss (Elias and Menon, 1991; Downing, 1992). Keratinocytes in the basal proliferating layer, as well as the differentiating cells of the spinous and granular layers, respond to regulatory signals to balance the processes of growth, differentiation, and apoptosis to maintain epidermal homeostasis (Fuchs, 1990). The anucleate corneocytes of the stratum corneum, which display a unique external envelope, the highly insoluble cornified envelope (CE), are the end product of epidermal differentiation. Epidermal differentiation comprises the sequential expression of keratins 1 and 10, profilaggrin, involucrin (INV), loricrin, and a variety of other proteins of the CE (Fuchs, 1990). The calcium-dependent, epidermal-specific enzyme, transglutaminase 1 (TG'ase), which is expressed late in differentiation, crosslinks these proteins into the CE (Rice and Green, 1979; Thacher, 1989; Dlugosz and Yuspa, 1994; Robinson *et al*, 1996).

Keratinocytes also sequentially express differentiation markers such as INV and TG'ase when cultured in media containing calcium (Hennings *et al*, 1989; Pillai and Bikle, 1991). In contrast, keratinocytes maintained in low concentrations of extracellular calcium (0.03 mM) remain in a proliferative state, and express very low levels of INV and TG'ase mRNA and protein, resulting in low rates of CE formation. Furthermore, incubation in 1.2 mM calcium, which results in increased intracellular calcium levels, stimulates the expression of these proteins and increases CE formation (Hennings *et al*, 1989; Dlugosz and Yuspa, 1994). While the INV gene contains calcium-responsive regions (Ng *et al*, 1996) and calcium stimulates the protein kinase C-activator protein 1 (AP-1) signaling pathways that regulate differentiation-specific gene expression (Banks *et al*, 1998; Rossi *et al*, 1998), the biochemical pathways regulating calcium-induced keratinocyte differentiation have not been fully elucidated.

Epidermis is also active in synthesizing lipids destined for specialized membrane complexes of the intercorneocyte spaces and

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Reprint requests to: Karen Hanley, Dermatology Service (190), Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121.

Abbreviations: CE, cornified envelope; INV, involucrin; KGM, keratinocyte growth medium; LXR, liver X-activated receptor; TG'ase, transglutaminase 1; 22R-OHCh, 22R-hydroxycholesterol; 25-OHCh, 25-hydroxycholesterol.

required for permeability barrier function. Several of these lipid species also regulate keratinocyte growth and differentiation. For example, ceramides induce differentiation and inhibit proliferation (Pillai *et al*, 1996; Geilen *et al*, 1997; Jung *et al*, 1998), whereas glucosylceramides stimulate proliferation (Marsh *et al*, 1995). Additionally, 1,25-dihydroxyvitamin D_3 and retinoids, ligands for nuclear receptors in the RXR-interacting family of nuclear hormone receptors (Kastner *et al*, 1995; Mangelsdorf *et al*, 1995), have well-known effects on keratinocyte differentiation and proliferation (Pillai and Bikle, 1991; Itin *et al*, 1994; Fisher and Voorhees, 1996). Furthermore, we have recently shown that long-chain fatty acids, activators of the peroxisome proliferator-activated receptor, induce keratinocyte differentiation and inhibit growth (Hanley *et al*, 1998). Thus, intracellular metabolites may regulate keratinocyte growth and differentiation via RXR-interacting nuclear hormone receptors.

In this study, we asked whether other endogenous lipid metabolites, such as oxygenated cholesterols, which activate liver X-activated receptor (LXR) α and β, members of the RXR superfamily (Mangelsdorf and Evans, 1995; Mangelsdorf *et al*, 1995; Willy *et al*, 1995; Janowski *et al*, 1996), regulate keratinocyte differentiation and growth. We show here that active LXR α and β are present in human keratinocytes, and that oxysterols induce the coordinate expression of the differentiation-specific genes INV and TG'ase, increase CE formation, and inhibit cellular proliferation. Moreover, the effects of oxysterols occur independently of calcium, but are synergistic to the well-known effects of this cation on differentiation. Furthermore, we show that oxysterol-induced transcription of the INV gene involves an AP-1 site (–2117 to –2111 bp) important for this induction. Together, these results point to another class of endogenous metabolites, oxygenated sterols, as potent transcriptional regulators of epidermal differentiation.

MATERIALS AND METHODS

Cell culture Human epidermis was isolated from newborn foreskins and keratinocytes were plated in serum-free keratinocyte growth medium (KGM) (Clonetics, San Diego, CA), as described by Gibson *et al* (1996). Cells were treated with either 22R-hydroxycholesterol (22R-OHCh), 25-hydroxycholesterol (25-OHCh), cholesterol, mevalonate (Sigma, St Louis, MO), or vehicle (0.05% ethanol) for 24 or 48 h, as indicated for each experiment. Oxysterols and cholesterol were dissolved in ethanol and stored at –20°C. Mevalonate was solubilized in sterile water.

RNA isolation, northern blotting and cDNA probes Total RNA was isolated by using Trizol reagent (Sigma) following the manufacturer's protocol. Poly(A)$^+$ mRNA was isolated as described by Harris *et al* (1998). Ethanol-precipitated RNA pellets were resuspended in sterile, DEPC-treated water, and RNA was quantified by absorbance at 260 nm. The 260:280 nm ratio was used as an index of purity. Total RNA (15 μg per sample) or poly(A)$^+$ mRNA (8 μg per sample) was size fractionated through a 1% agarose gel containing 2.2 M formaldehyde, as described previously (Hanley *et al*, 1998). RNA integrity was visualized following acridine orange staining of the electrophoresed gel. The RNA was transferred to a nylon membrane that was subsequently baked at 80°C for 2 h. Blots were hybridized with ^{32}P-labeled probe: INV (a gift from Dr. Howard Greene), keratinocyte TG'ase (a gift from Dr. Robert Rice), or hLXR α or β (Willy *et al*, 1995) overnight at 65°C. Blots were washed for 20 min at room temperature and then 20 min at 65°C as described previously (Hanley *et al*, 1998). Autoradiography was performed at –70°C. Blots were probed with β-actin to confirm equal loading. Appropriate bands were quantified by densitometry. Molecular biology-grade reagents were obtained from Sigma or from Pharmacia Biotech (Uppsala, Sweden).

INV and transglutaminase protein levels Protein concentration was assessed by protein electrophoresis and western blotting, as described previously (Hanley *et al*, 1998). Briefly, cells were lyzed in 2% sodium dodecyl sulfate (SDS) and the lysate was sonicated. Following protein determination (Bicinchoninic acid protein assay; Pierce, Rockford, IL), equal amounts of protein (50 μg) were electrophoresed on 7.5% polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (0.2 μm; Bio-Rad Laboratories, Hercules, CA). INV protein was detected by incubation overnight at 4°C with a polyclonal rabbit antihuman INV antibody (a gift from Dr. Robert Rice) (1:1000 in Tween 20, 0.5%, and nonfat dry milk, 5%). Densitometry was used to quantitate INV-specific bands on the autoradiograms. TG'ase protein expression was measured as for INV expression, with modifications as described previously (Hanley *et al*, 1998). Briefly, stacking, sample, and running buffers all contained 4 M urea, and following electrophoresis, gels were washed in 4 M urea, 25 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.1 mM dithiothreitol (DTT), and 2 mM ethylenediamine tetraacetic acid for 90 min prior to electroblotting, allowing TG'ase to be detected by BC.1 primary antibody (Thacher, 1989). Specific bands on autoradiograms were quantified by densitometry.

CE formation The rate of CE formation was determined as described previously (Hanley *et al*, 1998). Briefly, cells were labeled with ^{35}S-methionine/cysteine (2 μCi per ml; Trans ^{35}S label, ICN Biomedical, Irvine, CA) for 48 h, incubated for the last 2 h with 5 μM ionomycin (Sigma), washed with calcium- and magnesium-free phosphate buffered saline (CMF-PBS), and harvested into 1.1 ml of 2% SDS. One milliliter of lysate was sonicated briefly (10 s) and added to 1 ml of 4% SDS-4 mM DTT, heated to 95°C for 30 min, and cooled to approximately 20°C. SDS/DTT-insoluble material was collected on filter discs, washed with 0.5% SDS-0.5% DTT, and quantitated by scintillation spectrophotometry. To determine total protein synthesized during the 48 h of ^{35}S labeling, 20 μl of the cell lysate taken prior to heating was precipitated with an equal volume of 2% bovine serum albumin and 1 ml of 10% (wt/vol) trichloroacetic acid (TCA) on ice for 30 min, and ^{35}S-labeled precipitated protein was collected onto filters (pore size P8, Fisher Scientific, Pittsburgh, PA), washed with 5% TCA, and quantified by scintillation spectroscopy. Total protein was determined by the method of Bradford (Bio-Rad). The percentage of cornified envelopes was calculated as percent cpm: (^{35}S-labeled DTT- and SDS-insoluble material)/total protein cpm × 100.

DNA synthesis The rate of DNA synthesis was determined as described previously (Hanley *et al*, 1998) with minor modifications. Briefly, [^{3}H]thymidine incorporation into cellular DNA was measured after 16 h of incubation with 2 μCi [^{3}H]thymidine (110 Ci per mmol methyl, 1′2′-[^{3}H]thymidine, Amersham, Arlington Heights, IL) per ml media. Cells were solubilized in 1N NaOH, and the radioactivity in the washed TCA precipitate was quantitated by scintillation spectroscopy.

DNA constructs and polymerase chain reaction mutagenesis The 3.7 kb INV promoter was a gift from Dr. J. Carroll (State University of New York, Stony Brook, NY) and the 2.2 kb TG'ase promoter-luciferase construct was a gift from Dr. Robert Rice. Deletional INV constructs were generated as described previously (Ng *et al*, 1996). The INV construct containing the mutant AP-1 site was prepared by polymerase chain reaction, using oligonucleotides 5′-tcga-tatgccg-tgagtca-gagggc-3′ and 5′-tcga-tatgccg-tgagCca-gagggc-3′. The AP-1 site is underlined and the single bp mutation is in upper case.

Transfections Keratinocytes were transfected as described previously (Ng *et al*, 1996) with minor modifications. Briefly, keratinocytes were transiently transfected 1 d after plating (at a confluence of approximately 20%–40%). 10 μg per ml final concentration of polybrene (dihexabromide, Aldrich Chemical), RSV-β-galactosidase (0.2 μg), and either INV- or TG-promoter-luciferase construct (2 μg), 2 μg AP-1 (7×) luciferase construct (kindly provided by Tom Parks, Cellegy, San Francisco, CA), or 0.5 μg LXRE (TK-LXRE$_3$-luciferase), were added in media (KGM containing 0.03 mM Ca^{2+}) in a final volume of 0.65 ml. Keratinocytes were incubated at 37°C for 5 h with gentle shaking each hour. Cells were then rinsed with CMF-PBS, followed by incubation at room temperature for 3 min with 10% glycerol in media. Cells were again rinsed with CMF-PBS, incubated overnight with fresh KGM containing 0.03 mM Ca^{2+}, and then treated as indicated for each experiment. Cells were rinsed and harvested in 250 μl reporter lysis buffer (Promega, Madison, WI). The lysate was centrifuged at 10,000*g* (4°C) for 2 min, and 10–20 μl of supernatant assayed with luciferase substrate (Promega) and β-galactosidase substrate galacto-light (Tropix, Bedford, MA) following manufacturer's instructions. β-galactosidase activity was used to normalize data and correct for variations in transfection efficiencies.

Isolation of nuclear proteins, gel shift assay Nuclear proteins were prepared from preconfluent keratinocytes incubated in low calcium in the presence of 22R-OHCh, 25-OHCh, or vehicle for 24 h, using the method described by Dignam *et al* (1983). The AP-1 oligonucleotides used were 5′-TCGA-TATGCCG-*TGAGTCA*-GAGGGC3′ and 5′-TCGA-GCC-CCTC-*TGACTCA*-CGGGCATA-3′; AP-1 mutants were 5′-TCGA-

TATGCCG-*aactgCA*-GAGGGC-3′ and 5′-TCGA-GCCCCTC-*TGcagtt*-CGGGCATA-3′ (AP-1 sites are in italics; lower-case letters denote mutations). Double-stranded oligonucleotides were end labeled using 50 μCi of γ ^{32}P-ATP (3000 Ci per mmol) in the presence of T4 polynucleotide kinase (Amersham) for 60 min at 37°C and were purified using G-50 microcolumns (Amersham). Binding reactions were performed for 30 min at 4°C using 5 μg nuclear extracts, buffer (10 mM Tris(hydroxymethyl)-aminomethane, pH 7.5, 1 mM DTT, 1 mM ethylenediamine tetraacetic acid, 5% glycerol), 2 μg poly(dI-dC), and 60,000 cpm of labeled oligonucleotide in a final volume of 15 μl. Radioinert competitor DNA was added at a 100-fold molar excess. The samples were electrophoresed for 1.5 h on 5% acrylamide gels using a one-half × TBE running buffer, dried, and autoradiographed.

Statistics Statistical analysis was performed using a Student's t test.

RESULTS

INV and TG'ase mRNA levels are increased by oxysterols To determine whether oxysterols affect keratinocyte differentiation, we first measured INV and TG'ase mRNA levels by northern analysis in keratinocytes incubated in the presence of 25-OHCh, 22R-OHCh, cholesterol (chol), mevalonate (mev), or ethanol vehicle (veh). As shown in **Fig 1**(***A***), keratinocytes maintained in low calcium (0.03 mM) and treated for 24 h with either 25-OHCh or 22R-OHCh exhibited an approximately 2–2.5-fold increase in mRNA levels of both INV and TG'ase. Similar results were obtained after 48 h of treatment with either oxysterol (data not shown). In contrast, cholesterol had no effect on mRNA levels. The effect of oxysterols on INV and TG'ase mRNA levels was dose-dependent, as shown in **Fig 1**(***B***, ***C***). Maximal effects were seen with 10–15 μM 22R-OHCh or 25-OHCh and half-maximal effects occurred with approximately 5 μM, whereas toxic effects were noted at concentrations over 15 μM. Because cholesterol does not traverse cell membranes as readily as oxysterols, and thus intracellular cholesterol levels may not attain those of oxysterols, we also examined the effects of mevalonate, a cholesterol precursor, on INV and TG'ase mRNA levels. Similar to cholesterol, mevalonate, at concentrations of either 200 μM (**Fig 1***A*) or 500 μM (not shown), did not affect mRNA levels of either INV or TG'ase. These results show that oxygenated sterols, but neither cholesterol nor mevalonate, increase INV and TG'ase mRNA levels.

A shift from low (0.03 mM) to high (1.2 mM) extracellular calcium is well known to stimulate differentiation in cultured keratinocytes. To determine whether oxysterol treatment together with calcium might further stimulate differentiation, we next measured INV and TG'ase mRNA levels in keratinocytes incubated for 24 h in 1.2 mM calcium plus 22R-OHCh or vehicle alone. 22R-OHCh increased mRNA levels of INV and TG'ase approximately 2.5-fold over vehicle-treated high calcium controls (**Fig 2**). β-actin mRNA levels were unaffected by oxysterol treatment in either low or high calcium (data not shown). As shown in **Fig 2**, the effects of high calcium and 22R-OHCh appeared to be synergistic rather than additive. Finally, pretreatment of keratinocytes with cycloheximide (5 μg per ml), which inhibits new protein synthesis, blocked oxysterol induction of INV and TG'ase mRNA (data not shown). Thus, the stimulatory effect of oxysterols on mRNA levels of INV and TG'ase requires *de novo* protein synthesis. These data indicate that oxysterols stimulate mRNA levels of both INV and TG'ase independently of calcium, and in a manner that requires protein synthesis and is synergistic to high calcium conditions.

Protein levels of INV and TG'ase are increased by oxysterols To determine whether oxysterols also increase INV and TG'ase protein, we next measured INV and TG'ase protein levels in keratinocytes incubated in either low or high calcium and treated with either 25-OHCh or 22R-OHCh, cholesterol, or ethanol vehicle. As shown in **Fig 3**, both 25-OHCh and 22R-OHCh increased INV and TG'ase protein levels approximately 2–3-fold in keratinocytes incubated under low calcium conditions. High calcium alone stimulated the levels of these proteins approximately 2–2.5-fold, and treatment with oxysterols further increased protein levels (approximately 2.5–3.5-fold over high calcium controls). Thus, protein levels of INV and TG'ase are increased by oxysterols in keratinocytes in the presence of either low or high calcium.

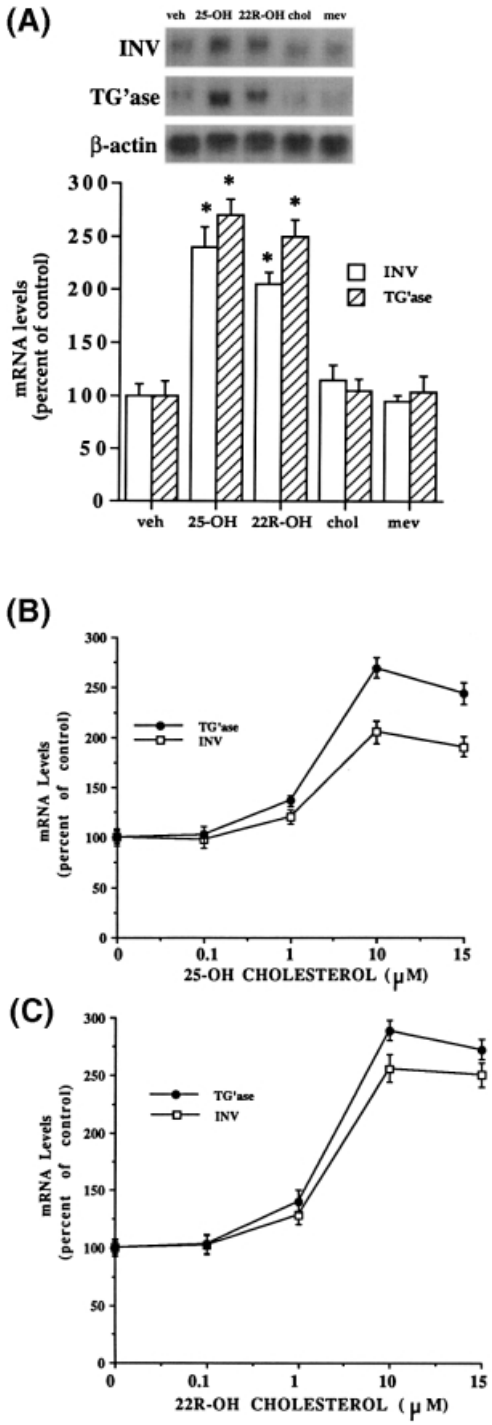

Figure 1. Induction of INV and TG'ase mRNA by oxysterols. (*A*) Keratinocytes were incubated in media containing 0.03 mM calcium in the presence of 0.05% ethanol vehicle (veh), 10 μM 25-OHCh, 22R-OHCh, or cholesterol (chol), or 200 μM mevalonate (mev). Fifteen micrograms total RNA was isolated and subjected to northern analysis as described in *Materials and Methods*. Data presented here (mean ± SEM) represent the average of three separate experiments; *$p < 0.01$. A representative autoradiograph is shown. (*B*, *C*) Keratinocytes were incubated 24 h in KGM containing 0.03 mM calcium and increasing concentrations of 25-OHCh (*B*) or 22R-OHCh (*C*). Graphs represent the average (mean ± SEM) of two separate experiments.

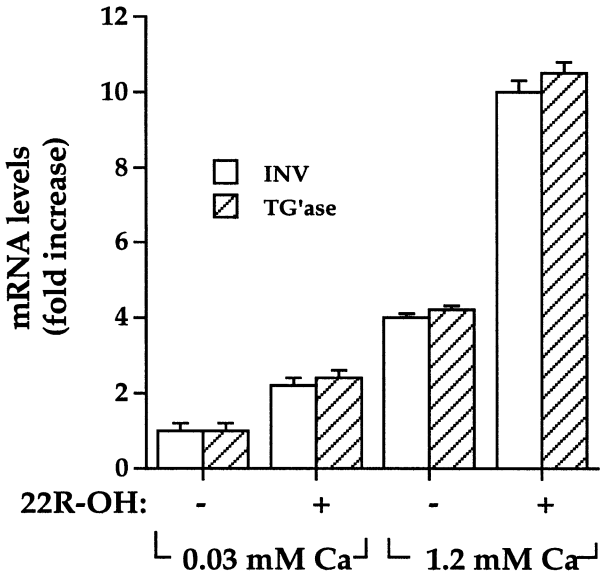

Figure 2. INV and TG'ase mRNA levels are increased by oxysterols in keratinocytes incubated in high calcium. Keratinocytes were incubated in either low (0.03 mM) or high (1.2 mM) calcium with or without 10 μM 22R-OHCh. RNA isolation and northern analysis was performed as described in the legend to **Fig 1**. Graphs represent the average of data from two independent experiments (two different donors).

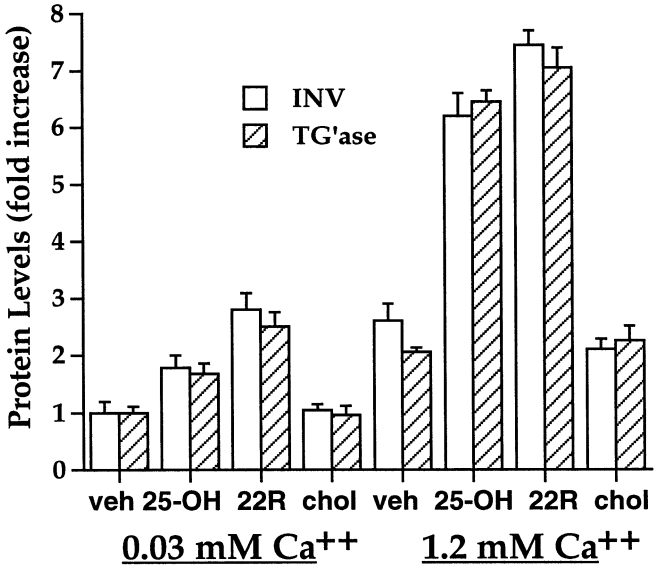

Figure 3. Protein levels of INV and TG'ase are increased by oxysterols in low calcium and in high calcium. Keratinocytes were incubated in the presence of 0.05% ethanol vehicle (veh), 10 μM 25-OHCh, 22R-OHCh, or cholesterol (chol), under either low (0.03 mM) or high (1.2 mM) calcium conditions. Western analysis was performed as described in *Materials and Methods*. The histogram represents the average (mean ± SEM) of data from two separate experiments (two different donors).

Rates of CE formation are increased by oxysterols The rates of CE formation were assessed as a marker of keratinocyte terminal differentiation. As shown in **Fig 4**, treatment with either 25-OHCh or 22R-OHCh resulted in a significant increase in rates of CE formation (140% increase by 25-OHCh and 160% by 22R-OHCh) in keratinocytes incubated in low calcium, conditions that normally suppress CE formation. The increase in CE formation by oxysterols (200% by 25-OHCh and 230% by 22R-OHCh), however, was more pronounced in high calcium, conditions

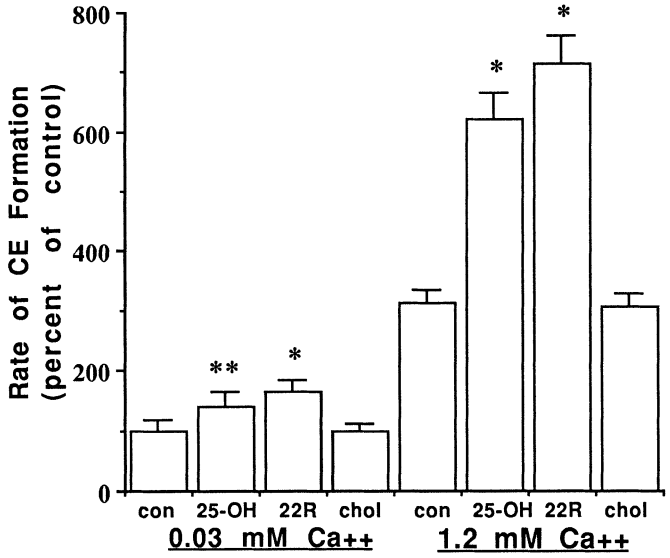

Figure 4. The rate of CE formation is increased by oxysterols in low or high calcium. Keratinocytes were incubated in media containing 0.03 mM or 1.2 mM calcium in the presence of 0.05% ethanol vehicle (con), 10 μM 25-OHCh, 22R-OHCh, or cholesterol (chol), together with ^{35}S-methionine/cysteine for 48 h. SDS/DTT-insoluble material was quantitated by scintillation spectrophotometry as described in *Materials and Methods*. Data presented here (mean ± SEM) represent the average of two separate experiments; $^{**}p < 0.05$; $^{*}p < 0.01$.

Table I. Inhibition of DNA synthesis by oxysterols[a]

Treatment	CPMS per μg DNA
Vehicle	100.0 ± 7.8
25-OHCh	51.3* ± 8.6
22R-OHCh	47.5* ± 6.2
Cholesterol	112.5* ± 9.8

[a]Preconfluent keratinocytes were treated for 24 h with vehicle (0.05% ethanol), 10 μM 25-OHCh, 22R-OHCh, or cholesterol. The rate of DNA synthesis was determined by measuring [^{3}H]thymidine incorporation into cellular DNA after 16 h of incubation with [^{3}H]thymidine as described in *Materials and Methods*. *p<0.01, n=3. Similar results were obtained in two separate experiments.

permissive for CE formation. In contrast, neither cholesterol (chol) (**Fig 4**), mevalonate (500 μM, data not shown), nor vehicle had an effect on CE formation in keratinocytes in either low or high calcium. These data indicate that oxysterols induce terminal differentiation in keratinocytes.

DNA synthesis is inhibited by oxysterols Oxysterols such as 25-OHCh are potent inhibitors of cell growth in other cell types such as thymocytes and lymphocytes (Christ *et al*, 1992; Ayala-Torres *et al*, 1997). To determine whether oxysterols also inhibit keratinocyte growth, we next measured rates of DNA synthesis in keratinocytes treated with 25-OHCh, 22R-OHCh, cholesterol, or vehicle alone. As shown in **Table I**, the rate of DNA synthesis was decreased in cells treated with oxysterols (55% of control by 25-OHCh and 49% by 22R-OHCh) during the 16 h time period measured. In contrast, neither cholesterol nor vehicle inhibited DNA synthesis. These results indicate that 22R-OHCh and 25-OHCh significantly inhibit keratinocyte growth.

Mevalonate does not reduce oxysterol-induced INV or TG'ase mRNA levels Oxysterols inhibit cholesterol synthesis by decreasing the activity of several key enzymes of cholesterol synthesis, including HMG CoA reductase. While exogenous cholesterol also inhibits cholesterol synthesis and HMG CoA

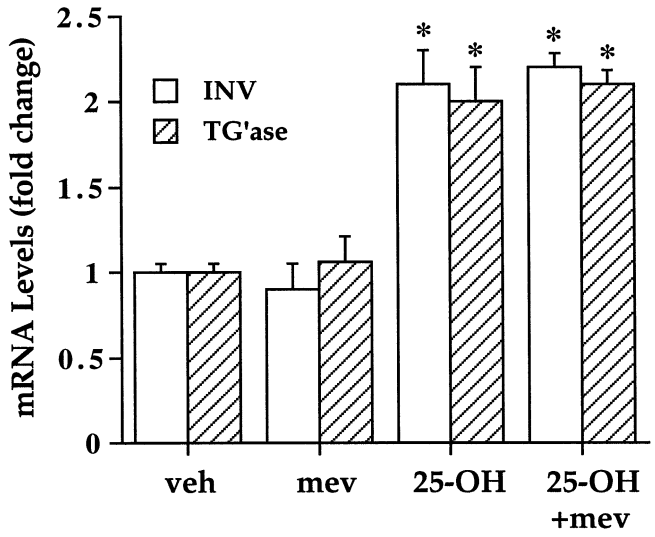

Figure 5. Mevalonate does not affect oxysterol induction of INV and TG'ase mRNA levels. Keratinocytes were treated with <0.1% ethanol vehicle (veh), 200 μM mevalonate (mev), 10 μM 25-OHCh, or mevalonate and 25-OHCh together (25-OH + mev). INV and TG'ase mRNA levels were measured as described in the legend to **Fig 1**. *$p < 0.01$. Similar results were obtained in two separate experiments.

reductase, 25-OHCh is a much more potent inhibitor (Brown and Goldstein, 1974). To determine whether the inhibition of HMG CoA reductase, which in addition to decreasing cholesterol levels leads to decreased levels of isoprenoids, might underlie the induction of INV and TG'ase mRNA levels by oxysterols, we next measured mRNA levels in keratinocytes treated with 25-OHCh in the presence or absence of mevalonate, the product of HMG CoA reductase. As shown in **Fig 5**, mevalonate alone again had no effect on INV or TG'ase mRNA levels, and 25-OHCh alone increased mRNA levels approximately 2-fold. Addition of mevalonate together with 25-OHCh had no effect on the increase in INV or TG'ase mRNA levels induced by 25-OHCh alone. These data suggest that a reduction in intermediates in the cholesterol synthetic pathway is not the basis for the increase in INV and TG'ase mRNA levels by oxysterols.

Regulation of the INV and TG'ase promoters by oxysterols Increased mRNA levels suggest regulation at the genomic level. To determine whether the increase in INV and TG'ase mRNA levels by oxysterols might be due to increased transcription, we transfected keratinocytes with either a 3.7 kb sequence of the INV promoter or a 2.2 kb sequence of the TG'ase promoter, both coupled to luciferase reporters. Reporter activity of both INV (**Fig 6*A***) and TG'ase (**Fig 6*B***) increased following treatment with 22R-OHCh or 25-OHCh (2–3-fold) compared with vehicle-treated controls.

Localization of oxysterol-responsive regions in the INV gene To localize oxysterol-responsive regions in the INV gene, we next transfected keratinocytes with a series of truncated or deletional INV promoter constructs, and then examined the effects of 22R-OHCh on promoter activity. Internal deletion of the intron at +182 bp to +1228 bp (construct designated 2.7 kb) or of the regions containing −1880 bp to −156 bp and −3 to +1228 bp (construct designated INV-P) had no effect on oxysterol responsiveness (**Fig 7*A***). In contrast, deletion of the region containing −2452 bp to −1880 bp resulted in loss of oxysterol responsiveness (**Fig 7*A***). Thus, the region spanning −2452 to −1880 bp of the INV promoter contains an oxysterol-responsive region.

A functional AP-1 site (AP1–5) has been identified within the −2452 to −1880 bp region of the INV promoter (Welter *et al*, 1995). Moreover, the AP-1–5 site mediates the increase in INV

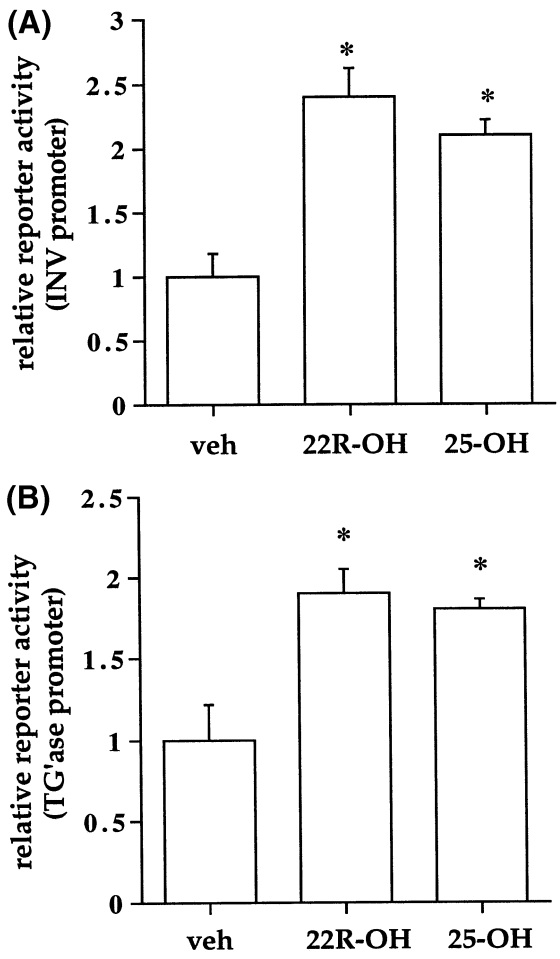

Figure 6. INV and TG'ase transcription is increased by oxysterols. Keratinocytes were transiently transfected with a 3.7 kb INV-luciferase construct (*A*) or a 2.2 kb TG'ase-luciferase construct (*B*) as described in *Materials and Methods*. Data presented here (mean ± SEM) represent the average of three separate experiments; *$p < 0.01$.

transcription following treatment with phorbol esters or calcium (Welter *et al*, 1995; Ng *et al*, 1996). To evaluate the role of this AP-1 binding site in the induction of INV transcription by oxysterols, reporter activity was measured in keratinocytes transfected with a construct containing a wild-type (WT) AP-1 response element (–2117 to –2111) or with a corresponding construct in which the AP-1 site was mutated (TGAGTCA mutated to TGAGCCA). As shown in **Fig 7(*B*)**, the oxysterol inducibility of reporter activity was abolished by the AP-1–5 site mutation.

Oxysterols increase AP-1 promoter activity and AP-1-DNA binding We next transfected keratinocytes with an AP-1 promoter construct coupled to a luciferase reporter, and compared the effects of oxysterols, cholesterol, or vehicle on reporter activity. As shown in **Fig 8(*A*)**, reporter activity was significantly increased by 22R-OHCh and 25-OHCh treatment and not affected by treatment with cholesterol. These data indicate that oxysterols increase AP-1-regulated transcription.

To determine whether oxysterols affect the binding of nuclear proteins to the AP-1–5 site in the INV regulatory region, we next performed gel shift and oligonucleotide competition experiments. **Figure 8(*B*)** shows results from experiments in which nuclear extracts from keratinocytes treated with 22R-OHCh or vehicle were incubated with ^{32}P-AP-1 in the presence or absence of excess radioinert AP-1 or mutant AP-1 competitor. Addition of nuclear extract to the AP-1 oligonucleotide resulted in a shifted band, and the intensity of this band shift was increased by oxysterol treatment. The addition of homologous oligonucleotides completely abolished

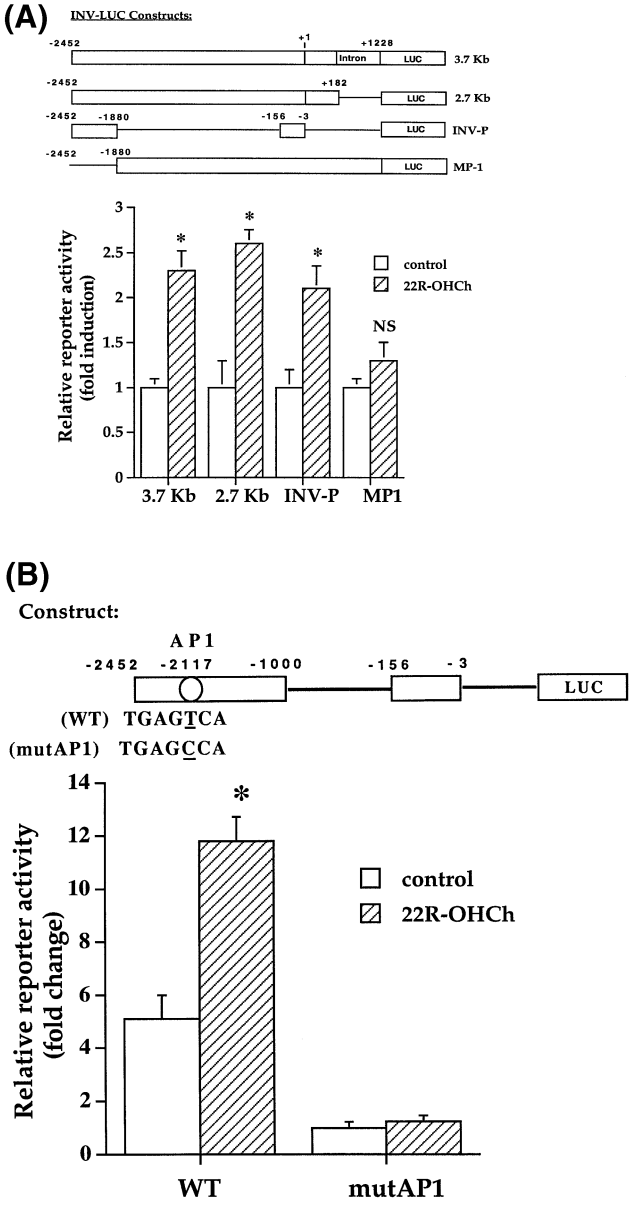

Figure 7. Effects of oxysterols on reporter activity in keratinocytes transfected with INV promoter-luciferase constructs. Keratinocytes were transiently transfected as described in *Materials and Methods*. Data presented here (mean ± SEM) represent the average of three separate experiments; *$p < 0.01$. (*A*) Cells were transfected with 2.0 μg of the corresponding INV-luciferase construct together with 0.2 μg RSV-β-galactosidase and were then incubated 24 h in the presence of vehicle (veh), 5 μM 22R-OHCh, or 25-OHCh. (*B*) Keratinocytes were transfected with an INV-luciferase construct containing either a wild-type (WT) or a mutated (mutAP-1) AP-1 site as described in *Materials and Methods*.

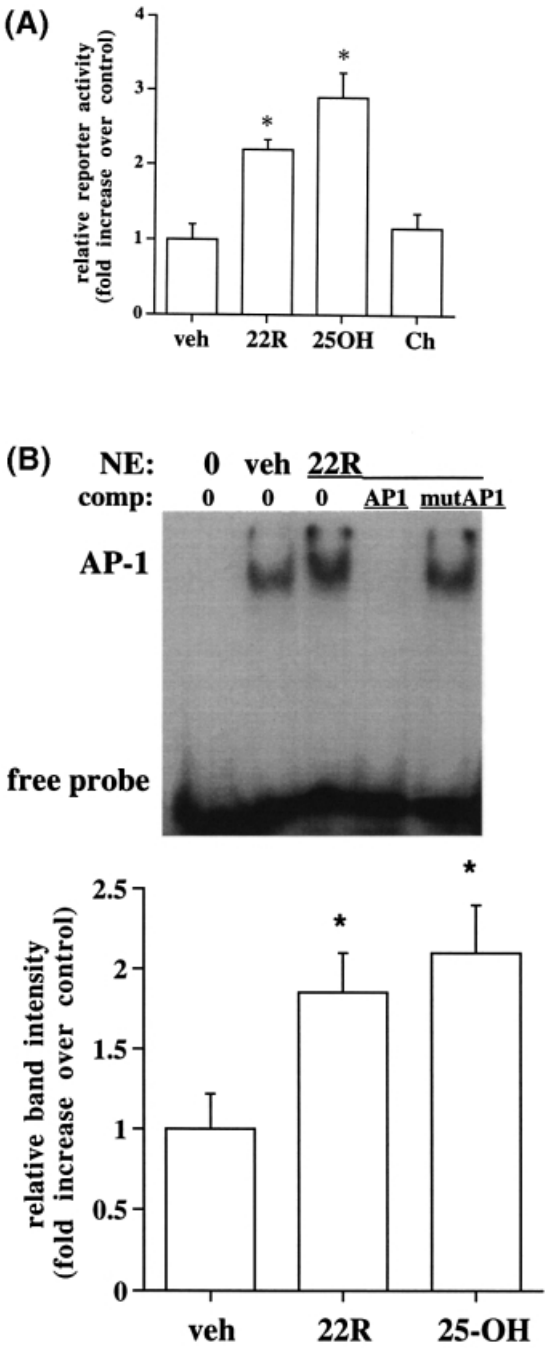

Figure 8. Increased AP-1 promoter activity and AP-1–DNA interactions by oxysterols. (*A*) Keratinocytes were transfected with 2 μg AP-1-luciferase construct together with 0.2 μg RSV-β-galactosidase, as described in *Materials and Methods*, and treated the following day for 24 h with vehicle (veh) (<0.05% ethanol), 5 μM 22R-OHCh, 25-OHCh, or cholesterol (Ch). (*B*) Keratinocytes were treated with vehicle (<0.05% ethanol) or 10 μM 22R-OHCh or 25-OHCh for 24 h and nuclear extracts (NE) were isolated as described in *Materials and Methods*. ^{32}P-AP1–5 was incubated with 5 μg NE in the absence or presence of 100-fold molar excess of AP-1–5 (AP1) or mutated AP-1–5 (mutAP1) competitors (comp). The incubations were electrophoresed on nondenaturing gels as described in *Materials and Methods*. A representative autoradiograph is shown here. Data (mean ± SEM) represent the average of three separate experiments; *$p < 0.01$.

binding to the probe, whereas addition of a mutated AP-1 resulted in no competition, suggesting that this band was the result of specific binding. As shown in the histogram (**Fig 8*B***), 25-OHCh treatment also increased AP-1-DNA binding. These data suggest that AP-1 binding factors are elevated in keratinocytes treated with oxysterols, thereby contributing to oxysterol-induced INV transcription.

Activation of an LXR response element and presence of LXR α and β in human keratinocytes To determine whether the effects of oxysterols on keratinocyte differentiation could be mediated by LXR, we next measured the activity of an LXR response element (LXRE) coupled to a luciferase reporter transfected into keratinocytes. Basal LXRE activity was present and fairly high (10,000 cpm per 20 μl, see *Materials and Methods*) in keratinocytes incubated in 0.03 mM calcium alone. The addition of either oxysterol resulted in modestly but significantly increased reporter activity (**Fig 9*A***). Further, the increase in reporter activity

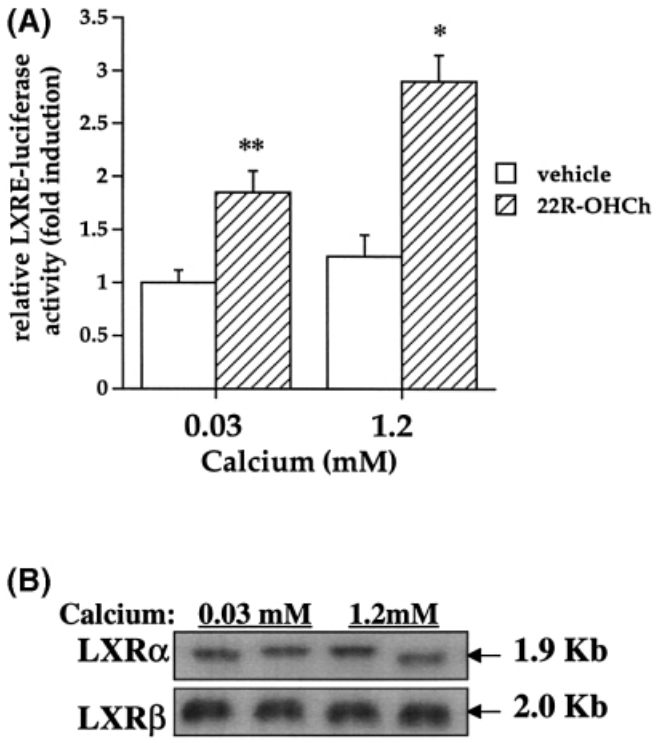

Figure 9. Increased LXRE activity by oxysterols and presence of LXR mRNAs. (*A***)** Oxysterols activate an LXR response element in human keratinocytes. Keratinocytes were cotransfected with TK-LXRE$_3$-LUC (0.5 μg) and RSV-β-galactosidase (0.2 μg) to correct for variations in transfection efficiency as described in *Materials and Methods*. Cells were then exposed to vehicle (< 0.1% ethanol) alone or 5 μM 22R-OHCh in low calcium (0.03 mM) or high calcium (1.2 mM) medium. Data are expressed as mean ± SEM, $^{**}p < 0.01$; $^{*}p < 0.005$. (*B*) Expression of LXR α and β mRNA in human keratinocytes cultured for 24 h in the presence of low calcium (0.03 mM) or high calcium (1.2 mM). Poly-A (+) mRNA was isolated as described in *Materials and Methods*. Eight micrograms was loaded per lane, northern analysis was performed, and blots were hybridized with either an LXR α or LXR β cDNA as described in *Materials and Methods*.

by oxysterols was more pronounced in keratinocytes incubated in the presence of 1.2 mM calcium (**Fig 9*A***).

The α isoform of LXR (RLD-1) has been demonstrated previously in whole adult rat skin (Apfel *et al*, 1994) and the β isoform (also named UR, OR-1) has been shown both in whole fetal rat skin and in human fibroblasts (Song *et al*, 1994; Teboul *et al*, 1995). We next determined whether either or both of these isoforms were present in human keratinocytes. Both LXR α and β were detected by northern analysis as transcripts of approximately 1.9 kb and 2.0 kb, respectively (**Fig 9*B***). mRNA levels of LXR α or β were comparable in 0.03 mM and 1.2 mM calcium (**Fig 9*B***).

DISCUSSION

This study demonstrates that oxysterols stimulate keratinocyte differentiation and inhibit proliferation. INV and TG'ase protein and mRNA levels, as well as a later marker of terminal differentiation, CE formation, are increased by exogenous oxysterols, both in cells incubated under proliferating (low calcium) conditions and in cells stimulated to differentiate by high calcium. Moreover, our transfection studies indicate that the increases in INV and TG'ase mRNA levels are due to increases in gene transcription. In contrast, cholesterol and mevalonate, a precursor of cholesterol, did not affect keratinocyte differentiation.

We have used a series of truncations and deletions of the INV promoter upstream of a luciferase reporter to identify an oxysterol-responsive region spanning nucleotides –2452 bp to –1872 bp. Furthermore, a single base pair mutation of the AP-1 site contained in this region (AP-1–5, –2117 to –2111 bp) abolished oxysterol responsiveness. Additionally, gel shift experiments revealed increased binding of the AP-1–5 to nuclear extracts from oxysterol-treated keratinocytes. Thus, this AP-1 site appears to be important for oxysterol-induced differentiation. It is of course possible that other regulatory sites in addition to this AP-1 site play a role in the oxysterol induction of INV expression.

The INV gene contains several AP-1 sites in the 5′-upstream portion of the promoter, two of which, the AP-1–5, and the AP-1–1 (–119 to –113 bp), have been shown to be essential for high level transcriptional activity. The effects of 12-O-tetradecanoylphorbol-13-acetate, a diacylglycerol analog that stimulates keratinocyte differentiation most probably through its activation of protein kinase C, are mediated via these AP-1 sites (Welter *et al*, 1995). All-*trans*-retinoic acid, which inhibits keratinocyte differentiation *in vitro*, inhibits many of the effects of phorbol esters mainly through antagonism of AP-1. Taken together, these data suggest that the AP-1 transcription factor positively regulates keratinocyte differentiation.

The region –2452 bp to –1872 bp has been found to be calcium-responsive and the AP-1 site contained within this region is essential for the calcium response (Ng *et al*, 1996). Because an increase in intracellular calcium is associated with keratinocyte differentiation (Hennings *et al*, 1989; Fuchs, 1990; Dlugosz and Yuspa, 1994), and some evidence suggests that vitamin D exerts its effects on keratinocyte differentiation by increasing calcium flux (Pillai and Bikle, 1991; Su *et al*, 1994), it is possible that calcium influx may be one of the signals for oxysterol-induced differentiation and inhibition of proliferation. In this study, however, we have shown that oxysterols increase INV and TG'ase mRNA even in the presence of concentrations of calcium that are maximally stimulatory, suggesting that oxysterols are not simply acting by altering calcium influx.

There are a number of mechanisms by which oxysterols could stimulate keratinocyte differentiation and inhibit proliferation. Oxysterols activate LXR α and LXR β, members of the RXR superfamily that regulate the metabolism of lipids including cholesterol and that have recently been demonstrated in skin (Apfel *et al*, 1994; Song *et al*, 1994; Teboul *et al*, 1995). Several other nuclear hormone receptors have been shown to alter transcription in other cell types through the AP-1 response element, and their ligands can influence each other's ability to modulate transcription through the AP-1 site (Uht *et al*, 1997). In this study we demonstrate the presence of both LXR α and LXR β mRNA in human keratinocytes. Moreover, we have shown high levels of basal activity of an LXR response element transfected into keratinocytes that are further increased by oxysterol treatment. Thus, the transcriptional machinery necessary for LXR to mediate keratinocyte differentiation is functional in keratinocytes. Whether oxysterols increase keratinocyte differentiation by activating LXR α and/or LXR β and whether these ligand-activated receptors interact with the AP-1 protein complex directly, or perhaps interact with transcription factors such as SP-1 that may in turn affect AP-1 binding, remains to be determined.

Oxysterols may affect the activity of other transcription factors. SF-1 is a member of the nuclear hormone receptor family, which in CV-1 cells has been reported to be activated by oxysterols (Lala *et al*, 1997). In steroidogenic cells, however, 25-OHCh does not affect the transcriptional regulation of SF-1-responsive genes (Mellon and Bair, 1998), and the expression of SF-1 is restricted to endocrine tissues, such as the adrenal gland, pituitary gland, and ovary (Luo *et al*, 1995). Therefore it is unlikely that oxysterols induce keratinocyte differentiation by activating SF-1. Oxysterols also inhibit the proteolytic conversion of SREBP-1 and SREBP-2 from an inactive 125K protein bound to the endoplasmic reticulum to a 65K protein that can migrate to the nucleus and regulate cholesterol and fatty acid metabolism by stimulating the expression of a number of genes

including the LDL receptor, HMG CoA synthase, FAS, acetyl CoA carboxylase, and HMG CoA reductase (Brown and Goldstein, 1997). Our laboratory has recently shown that SREBP-2 but not SREBP-1 is present in epidermis and in keratinocytes, and that changes in SREBP-2 levels regulate lipid synthesis (Harris *et al*, 1998). It is unlikely, however, that inhibition of SREBP activation could account for the oxysterol stimulation of keratinocyte differentiation because the addition of either cholesterol or mevalonate, both of which inhibit SREBP activation, did not stimulate keratinocyte differentiation. Finally, if keratinocyte differentiation was stimulated secondary to a deficiency of either cholesterol or isoprenoids, this should have been prevented by the addition of mevalonate. In this study, the addition of mevalonate simultaneously with oxysterols did not affect oxysterol-induced keratinocyte differentiation.

Cholesterol synthesis is very active in the epidermis (Feingold, 1991). Cholesterol is converted to oxysterols by P450 enzymes, which are expressed in many tissues including the epidermis (Jugert *et al*, 1994; Okuda, 1995; Keeney, 1998; unpublished observations). For example, 27-cholesterol hydroxylase is a widely expressed enzyme that oxidizes cholesterol (Anderson *et al*, 1989), which we have shown to be expressed in the epidermis (unpublished observations). Moreover, stresses to the epidermis such as ultraviolet light and air pollution, and chronological aging, increase the oxidative state of keratinocytes, thereby leading to increased formation of oxysterols (Ozawa *et al*, 1991; Darr and Fridovich, 1994; Fuchs *et al*, 1995). In tissues where it has been analyzed (liver, brain, adrenal) oxysterols are present in concentrations sufficient to activate LXR (Janowski *et al*, 1996; Smith, 1996; Lehmann *et al*, 1997). In the epidermis and keratinocytes, the levels of specific oxysterols have not yet been precisely determined.

In conclusion, this study demonstrates that naturally occurring oxysterols stimulate keratinocyte differentiation and inhibit proliferation. The mechanisms that mediate these responses to oxysterols are unresolved, but, as other nuclear receptors have been shown to be important regulators of keratinocyte differentiation and proliferation, activation of LXR α and/or LXR β could underlie these changes. Regardless of mechanism of action, oxysterols may prove to be of therapeutic value in the treatment of cutaneous disorders associated with decreased differentiation and increased proliferation, such as psoriasis.

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